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Abstract. We are examining apoptotic pathways that may be altered in human mammary epithelial cells as a consequence of the overexpression of prostaglandin G/H synthase 2. This project involves quantifying the apoptotic response as well as apoptotic regulators (ATM, MDM2, p53, bcl-2, and bax) in this cell system following exposure to agents that can induce apoptosis. We have shown that human mammary epithelial cells express high levels of prostaglandin G/H synthase, are resistant to ionizing radiation-induced apoptosis, but do apoptose when exposed to the broad spectrum kinase inhibitor staurosporine. This report documents progress made in the past year in measuring the apoptotic response in human mammary epithelial cells, in measuring the protein levels of some apoptotic regulators, and in the creation of human mammary epithelial cells that have attenuated levels of prostaglandin G/H synthase.

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FOREWORD

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Table of Contents:	Page
1. Front Cover	1
2. SF 298	2
3. Foreword	3
4. Table of Contents	4
5. Introduction	5
6. Body	8
7. Conclusions	10
8. References	11
9. Appendices	13

TITLE. The Role of Prostaglandin G/H Synthase 2 in the Apoptosis of Human Mammary Epithelial Cells

PROPOSAL RELEVANCE:

An understanding of early stages of cancer development is essential due to the correlation between patient survival and early detection, and investigation of the initial stages of cancer may suggest novel markers for patient screening or targets for new therapeutics. The majority of research on apoptosis has been carried out in hematopoetic or fibroblast cell lines, however, it is becoming increasingly evident that there may be important distinctions in how breast epithelial cells modulate their response to DNA damage or other insults. Since the majority of human cancers are derived from epithelial cells, the characterization of apoptosis, which may remove cells with intrinsic genomic instability, in this specific cell type should provide important information on the etiology of human breast cancer. Establishment of an epithelial tissue culture system was an important step toward characterizing this cell type. Breast epithelium normally undergoes cyclic apoptosis during the menstrual cycle, and a potential deregulation of this normal physiological occurrence could lead to the development of neoplasia. Immortalization itself may be an early event in the development of cancer, providing a pool of cells with altered proliferative capacity. We have available a normal human mammary epithelial cell line as well as an immortalized, non-tumorigenic, cell line derived from the normal cell line through treatment with the chemical carcinogen benzo(a)pyrene. The use of benzo(a)pyrene-immortalized mammary cells is an important distinction in these studies: these cells present a more realistic representation of developing breast neoplasms than virally-immortalized cell lines.

The research that is outlined in this proposal will also be the focus of my doctoral thesis proposal. I intend to pursue an independent career in research and would like to be able to focus on the development of breast cancer. My ultimate objective is to be able to operate my own laboratory where I may have the time and resources to conduct novel studies on the early stages of breast cancer, with a focus on how lipid metabolism may be a part of the carcinogenic process. In order to pursue this course of action, it is necessary to obtain a Ph.D. in order to be qualified for employment and funding opportunities from NIH and other agencies. The training that would be a direct result of the research in this proposal would allow me to hone the skills I have obtained to date, as well as learn new techniques. This proposal also sets the stage for additional studies. We have available fibroblasts from the same patient that the normal human mammary epithelial cell strain (HMEC184) was isolated from. Therefore we also have the capacity to investigate cell-type specific differences in the apoptotic pathways we will be characterizing in the HMEC. In addition, there are available extended lifespan variants of the HMEC184 cell strain that may possess an intermediate phenotype between normal and immortalized cells. I believe the field I have selected to study is a rapidly growing one: eicosanoids have long been suspected of playing a role in physiology other than the traditional inflammation response, and recently data has started to collect to elucidate some of these responses. This proposal will attempt to further characterize these signaling pathways that may be an early stage in the development of breast cancer.

BACKGROUND. Cancer cells can be distinguished based on their lack of differentiation, inappropriate proliferation, and immortal phenotype. The development of a cancer is a multi-step, lengthy process and can be separated into three steps; initiation, promotion, and progression. The first step, initiation, occurs when a DNA damaging agent causes a mutation that is fixed into the genome as the cell replicates. Promotion is a complex process, but it clearly involves a clonal expansion of initiated cells that have developed a selective growth advantage. The last step, progression, is a neoplastic evolution driven by genomic instability and resulting in eventual metastasis of the cancer cells. However, normal cells possess mechanisms to prevent the development of cancer. One important component in this defense is the ability of cells to control their progression

through the cell cycle. A G1 checkpoint allows cells to repair DNA damage before it becomes incorporated into the genome during S phase as a mutation. Alternatively, cells that have extensive damage or have bypassed the normal cellular checkpoints may be removed via the apoptotic pathway. Apoptosis is often referred to as "programmed cell death" and involves selective removal of cells. Characteristic morphological changes seen in apoptosis are a loss of cell volume, condensed chromatin, and plasma membrane blebbing. The nucleus breaks up into membrane-enclosed fragments, followed by the cell itself. The membrane-bound fragments are rapidly phagocytosed by the surrounding cells without triggering an inflammatory response.

Apoptosis is also an important mechanism in normal developmental processes such as embryogenesis, the immune response, and hormone-dependent structural changes in the breast and prostate. In the embryo, apoptosis acts to remove selected subpopulations of cells in various organs. This process removes autoimmune T lymphocytes during thymocyte maturation in the thymus and a large number of neurons in the developing brain (reviewed in (1)). In the mature organism, cytotoxic T cells are thought to kill some virally infected cells via apoptosis (2, 3), and the depletion of $CD4^+$ T cells during HIV infection may be due to uncontrolled apoptosis (4, 5). Following castration, the rat prostate undergoes involution and this process requires the programmed cell death of androgen-deprived cells (6). Apoptosis is a normal event in the lactating mammary gland (7) and also serves to remodel breast tissue following weaning (8, 9). Moreover, in normal breast tissue apoptosis shows a cyclic trend, peaking near the end of the menstrual cycle (10).

Proteins which mediate the apoptotic response in the breast include p53, bcl-2 and bax. p53 is a transcription factor that has been shown to inhibit cell proliferation and suppress transformation and tumorigenesis. Perturbation of p53 signaling pathways may give affected cells a growth advantage and contribute to neoplasia: accordingly p53 is mutated or lost in over 50% of human cancers. It has also been suggested that loss of wild-type p53 could contribute to the formation of radio- and chemotherapy-resistant tumors. Nelson and Kastan (11) found that DNA strand breaks are sufficient for increasing p53 protein levels, and Kastan *et al.*. (12)have demonstrated that fibroblasts from p53-null mice lack a G1 checkpoint induced by ionizing radiation. It has been suggested that p53 may also be involved in a G2 delay (13), although this may not be a direct effect (14). One negative regulator of p53 is the proto-oncogene MDM2, which was identified by its ability to enhance tumorigenic potential when overexpressed (15). It was demonstrated by Momand *et al.* (16) that p53-mediated transcriptional activation can by inhibited by mdm2 oncogene product binding to the p53 protein. Mdm2 mRNA levels can be induced by wild-type p53 (17) and this may form a negative feedback loop, whereby excessive p53 levels lead to an increase in mdm2 protein production and subsequent inactivation of the p53 protein (18). As would be expected, DNA damage can cause induction of Mdm2 in a p53-dependent fashion (19, 20). Another apparent modulator of p53 signaling is the ataxia telangiectasia (AT) gene product. AT is an autosomal recessive disease that is marked by hypersensitivity to ionizing radiation, radioresistant DNA synthesis, and a progressive cerebellar ataxia with degeneration of Purkinje cells. There is also an increase in cancer risk associated with AT , and women who are heterozygous for AT have up to a five-fold greater risk for breast cancer (21). The ionizing radiation induced increase in p53 levels is delayed in cells from AT patients (12). In addition, other components of the p53-dependent DNA damage response are also altered in AT cells: Waf1, Mdm2 and Gadd45 all exhibit delayed or decreased induction in AT cells exposed to ionizing radiation (22) A gene mutated in all four complementation groups of AT was recently identified. and a partial cDNA clone isolated (23).

Bcl-2 was identified ten years ago as a gene overexpressed in human follicular lymphoma due to a translocation event that places it under the influence of regulators of the IgH gene (24, 25). In normal human breast tissue, bcl-2 protein levels vary throughout the menstrual cycle and are highest at the end of the luteal phase (26). It is also commonly

overexpressed in breast cancer (27). Bcl-2 can also protect cells exposed to apoptosis-inducing stimuli, not all of which are p53-dependent (28). In cultured human mammary epithelial cells, bcl-2 overexpression inhibits cell death due to confluence or serum starvation (29), while overexpression of Bax accelerates apoptotic death. The activity of bcl-2 is thought to be mediated through a protein-protein interaction with bax, which is a member of the same protein family. Bax forms homodimers or heterodimers with bcl-2 *in vivo*, and it has been suggested that the bcl-2/bax ratio may act as a determinant of apoptosis (30). Supporting this theory is the finding that human breast cancer cell lines that are resistant to apoptosis contain a low level of bax-α (a splicing variant of bax) when compared to non-malignant epithelial cell lines (31).

Another potential mediator of programmed cell death in human breast cells is $p21^{Waf1/Cip1}$. $p21^{Waf1/Cip1}$ is a 21 kDa protein that can be induced in a p53 dependent manner in G1 arrest through inhibition of cyclin-dependent kinases (32) and apoptosis(33). Overexpression of $p21^{Waf1/Cip1}$ in human breast cancer cells results in morphological changes and induces growth arrest and apoptosis (34). $p21^{Waf1/Cip1}$ expression can be induced by a variety of agents including UV(35), oxidative stress (36), and kinase inhibitors (37). However, not all of these responses are p53 dependent (36, 38, 39).

Recently it has been shown that an enzyme involved in the inflammation process may also have an effect on cell cycle and apoptosis. Prostaglandin G synthase (also referred to as cyclooxygenase) and prostaglandin H synthase are two enzymatic reactions catalyzed by the same protein, a homodimer of approximately 70 kDa subunits (hereafter referred to as PGHS). PGHS catalyzes the conversion of arachidonic acid to the hydroperoxide PGG_2, then the reduction of PGG_2 to the alcohol PGH_2. Inhibitors of PGHS can decrease the risk of colon cancer (40), and can also reduce the number and size of adenomatous polyps (41). Elevated levels of prostaglandins are found in both lung and colon cancers (42, 43), and the mitogen-inducible form of prostaglandin G/H synthase (PGHS2) is expressed at high levels in a large number of colon cancers (44, 45). Overexpression of PGHS2 increases resistance to apoptotic stimuli in rat intestinal epithelial cells with a concurrent increase in bcl-2 levels (46), and alterations in PGHS2 expression may also affect the cell cycle (47). The PGHS enzymes may play a more indirect role in carcinogenesis: they can also metabolize the procarcinogen benzo[a]pyrene to the active carcinogen benzo[a]pyrene-7,8-diol-epoxide(48, 49, 50, 51).

The metabolic activation of benzo[a]pyrene (B[a]P) by human mammary epithelial cells (HMEC) produces not only B[a]P-DNA adducts, but also oxidative DNA damage (52). In normal HMEC184 cells, the production of this oxidative damage is transient and returns to undetectable levels when B[a]P treatment is ended. We have found that the induction of the oxidative DNA damage occurs via the arachadonic acid cascade. Treatment of cells with B[a]P in the presence of inhibitors of PGHS, indomethacin or ibuprofen, reduces the levels of one type of oxidative base modification, thymine glycols [redacted]. A human mammary epithelial cell line immortalized by multiple treatments with B[a]P, HMEC184B5, maintains elevated levels of thymine glycols even in the absence of B[a]P treatment. The basal levels of thymine glycols in these cells is equivalent to the cell receiving between 30-40 Gy of ionizing radiation. The production of the reactive oxygen species in these cells is also dependent on the arachadonic acid cascade since treatment with either indomethacin or ibuprofen significantly reduces the amount of thymine glycols (Table 1). The high background of oxidative DNA damage in the immortalized HMEC184B5 cells was not due to slower removal of this base damage relative to the normal HMEC184 cells. When thymine glycols are induced by treatment with hydrogen peroxide, both HMECs removed thymine glycols with a similar efficiency (data not shown). We investigated whether the high levels of thymine glycols in the immortalized breast cells could be due to the induction of the pathway which induces this oxidative DNA damage when the cells are treated with B[a]P, namely the arachadonic acid cascade. We

examined the expression of the inducible form of PGHS, PGHS2, in the immortalized HMEC184B5 cells. We found that there are high levels of both PGHS2 mRNA and protein in the HMEC184B5 cells relative to the normal HMEC184 cells. Therefore, it appears that the high levels of oxidative DNA damage induced in the immortalized breast cells is due to overexpression of PGHS2.

Since high levels of PGHS2 can protect rat intestinal epithelial cells from apoptosis(46), one prediction would be that the immortalized HMEC would be more resistant to apoptosis-inducing agents than normal HMEC, which do not possess high levels of PGHS2. We exposed HMEC184B5 cells to ionizing radiation to induce apoptosis. Apoptosis was measured by visualizing the production of a laddering of DNA fragments 180 base pairs or multiple in size, which is characteristic of apoptosis. Following treatment with either 8 or 16 Gy of ionizing radiation, no induction of DNA ladders was detectable by either 24 or 46 hours post-treatment. However, when HMEC184B5 are exposed to increasing doses of staurosporine, a protein kinase inhibitor shown to induce apoptosis, DNA fragmentation is detected. Our results with ionizing radiation suggest that that HMEC184B5 cells cannot undergo apoptosis via a p53-dependent pathway. However, DNA damage-induced apoptosis is not necessarily always mediated through p53(28). By comparison, staurosporine appears to act through a p53-independent pathway (53). Staurosporine, a non-specific serine/threonine kinase inhibitor, can disrupt the cell cycle and is thought to cause apoptosis by interfering with the timely activation of cell cycle components (54). Treatment of cells with staurosporine can lead to induction of $p21^{Waf1/Cip1}$ (37).

HYPOTHESIS/PURPOSE

Our preliminary results show that immortalized HMEC184B5 cells express high levels of PGHS2. In addition, these cells maintain extremely elevated levels of oxidative DNA damage, a situation one would expect to lead to apoptosis, yet appear to be unable to undergo apoptosis when exposed to ionizing radiation. **We hypothesize that the overexpression of PGHS2 prevents immortalized mammary epithelial cells from undergoing apoptosis via a p53-dependent pathway.** If our hypothesis is correct, then we expect that immortalized cells will not undergo apoptosis when exposed to agents which induce apoptosis via a p53-dependent pathway. Conversely, these cells should be able to apoptose when exposed to agents which induce apoptosis via a p53-independent pathway. We also expect the p53-independent induction of apoptosis to occur at a different stage in the cell cycle than does the p53-dependent pathway. Finally, we expect that either eliminating or reducing PGHS2 activity in the immortalized cells should restore their ability to undergo apotosis.

PROGRESS REPORT

We would like to report progress in the following areas:

Task 1-1: Training on use of the fluorescence microscope is complete. We have been successful in obtaining good phase-contrast (figure1) and fluorescence (figure 2) images from HMEC undergoing apoptosis, but the utility of this method for accurate analysis is still uncertain. However, the morphological measurements of apoptosis may not be quantitative, due to late-stage apoptotic cells releasing from the cell culture plate and becoming suspended in the media. A possible solution is to use morphological examination for positively classifying cell death as apoptotic, and relying on FACS analysis for quantification (as described below).

Recent developments have made the necessity of designing ATM and MDM2 probes unnecessary. Antibodies to the protein products of ATM and MDM2 are commercially available and have been used successfully by others in this laboratory. Therefore, Northern analysis of these potential apoptosis regulators will be replaced with Western analysis.

We have partially completed the dose response and time course of staurosporine-induced apoptosis. There have been difficulties in reproducibility using the apoptosis kit

from Boehringer Mannheim. This may be due to the kit design that lacks a method to normalize results. We have attempted to work around this by using two separate methods of quantifying apoptosis. First, we have begun to use an anti-PARP western blot method to measure activation of caspases, an early indicator of apoptosis (figure 3). PARP (poly(ADP-ribose) polymerase) is a DNA associated protein that undergoes cleavage by caspases early in the apoptotic process. Secondly, we have used FACS analysis to quantify DNA fragmentation and the loss of cell volume concurrent with apoptosis. This technique is particularly useful because it is a direct measurement of cell numbers. It also provides useful information on how cell death relates to the cell cycle. For example, we can detect a replication block in the normal and immortalized HMEC exposed to 2.0 uM staurosporine after 6 hours, indicated by a reduction in BrdU incorporation, as well as a sub-G1 apoptotic population detected at 12 hours in the HMEC 184 cells and 6 hours in the HMEC 184B5 cells (figures 4 and 5). Surprisingly, the immortalized HMEC 184B5 cells appear to undergo apoptosis sooner than do the normal HMEC184 cells. This is in contrast to the data we obtained from a dose response taken at 24 hours post-treatment. Figures 6 and 7 demonstrate that the normal HMEC 184 cells respond to staurosporine by undergoing a replication block (seen as a reduction of cells in S phase) at a lower dose than do the immortalized HMEC 184B5 cells. An increase in sub-G1 population is seen with both increasing staurosporine dose, and with time after exposure to 2uM staurosporine.

The analysis of apoptotic regulators in HMEC exposed to staurosporine is also still underway. We have not had much success in probing for MDM2 and ATM as of yet, however, several persons in this laboratory have used these antibodies successfully on this cell type. We are currently entertaining helpful suggestions from these people. We do not see a significant increase in p53 in HMEC exposed to various doses of staurosporine at the 24 hour mark (figure 3A), however bcl-2 levels do decrease in a time-dependent manner following exposure of HMEC 184B5 cells to 2.0uM staurosporine (figure 3B). Bax levels do not fluctuate, but this may be unnnecessary since the bax/bcl-2 ratio is influenced by the drop in bcl-2 levels.

Task 2-1: We are please to report that, ahead of schedule, we have begun development of human mammary epithelial cells that contain an antisense-expressing construct to attenuate the high levels of endogenous prostaglandin G/H synthase 2. HMEC 184B5 cells were transfected with either a control vector (pCB7) or a vector containing the cDNA sequence of prostaglandin G/H synthase 2 in the antisense orientation, driven by a CMV promoter and a hygromycin B selection cassette (pCB7COX2AS). Several hygromycin-resistant clones for each transfection were isolated, and are now being expanded for freezedown and analysis. The clones isolated thus far show no aberrant morphology or growth characteristics when compared to wildtype HMEC 184B5 cells.

CONCLUSIONS:

We have drawn the following conclusions after a year of work on this project:

1) Human mammary epithelial cells maintain an intact apoptotic pathway that can be induced with the protein kinase inhibitor staurosporine;
2) Apoptosis can be induced in a time- and dose- dependent manner in both normal and immortalized HMEC;
3) The higher levels of prostaglandin G/H synthase in the immortalized HMEC 184B5 cells do correlate with the onset of apoptosis;
4) The immortalized HMEC 184B5 cells do require a higher dose of staurosporine to exhibit a replication block at 24 hours than do the normal HMEC 184 cells;
5) HMEC 184B5 cells show decreasing bcl-2 levels with time following exposure to staurosporine;
6) p53 levels do not appear to change in normal or immortalized HMEC following exposure to staurosporine.

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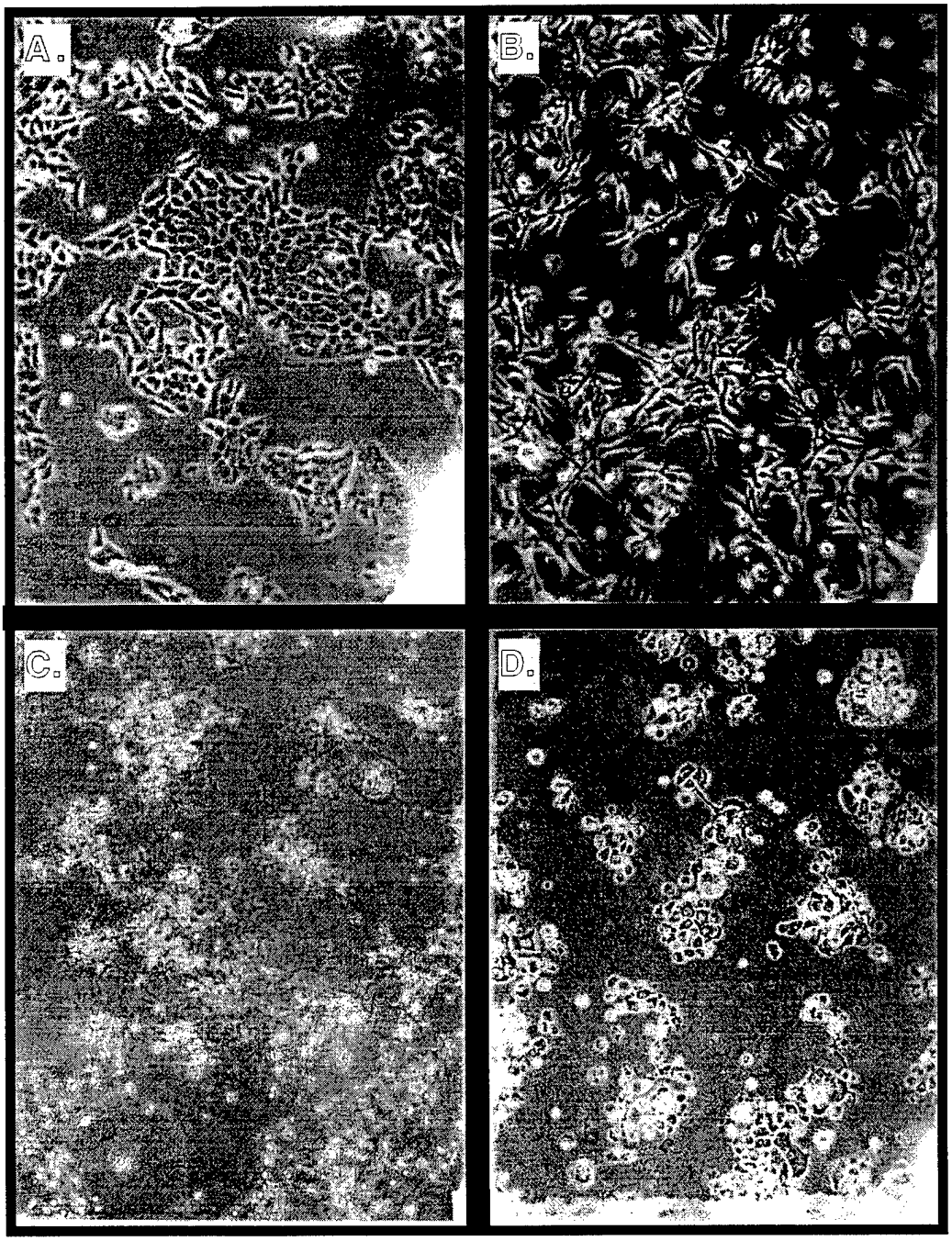
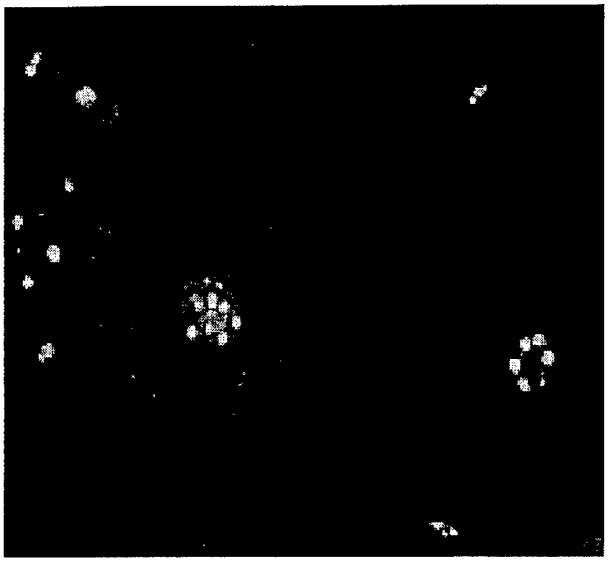


Figure 1 Morphological changes induced by staurosporine treatment. HMEC184B5 cells were treated for 24 hrs with **A**) 0.9% DMSO, **B**) 0.02 μM, **C**) 0.2 μM, or **D**) 2.0 μM staurosporine.

A.



B.

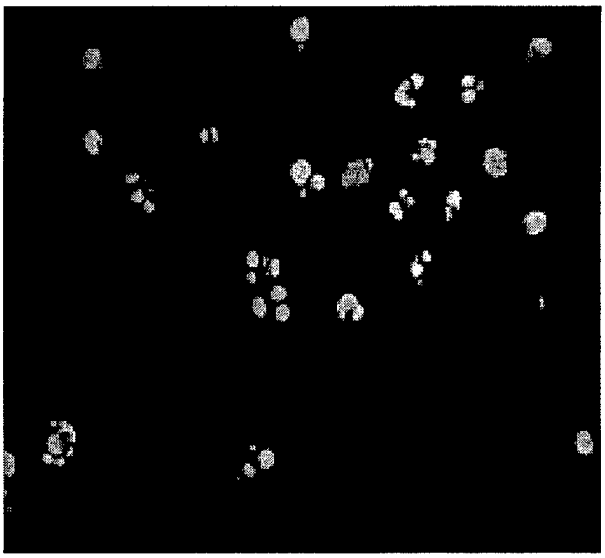


Figure 2 HMEC exhibit morphology characteristic of apoptosis following treatment with staurosporine. HMEC184 cells (A.) or HMEC 184B5 cells (B.) were treated with 2.0 mM staurosporine for 24 hours, fixed, stained with propidium iodine, and then visualized via florescence microscopy.

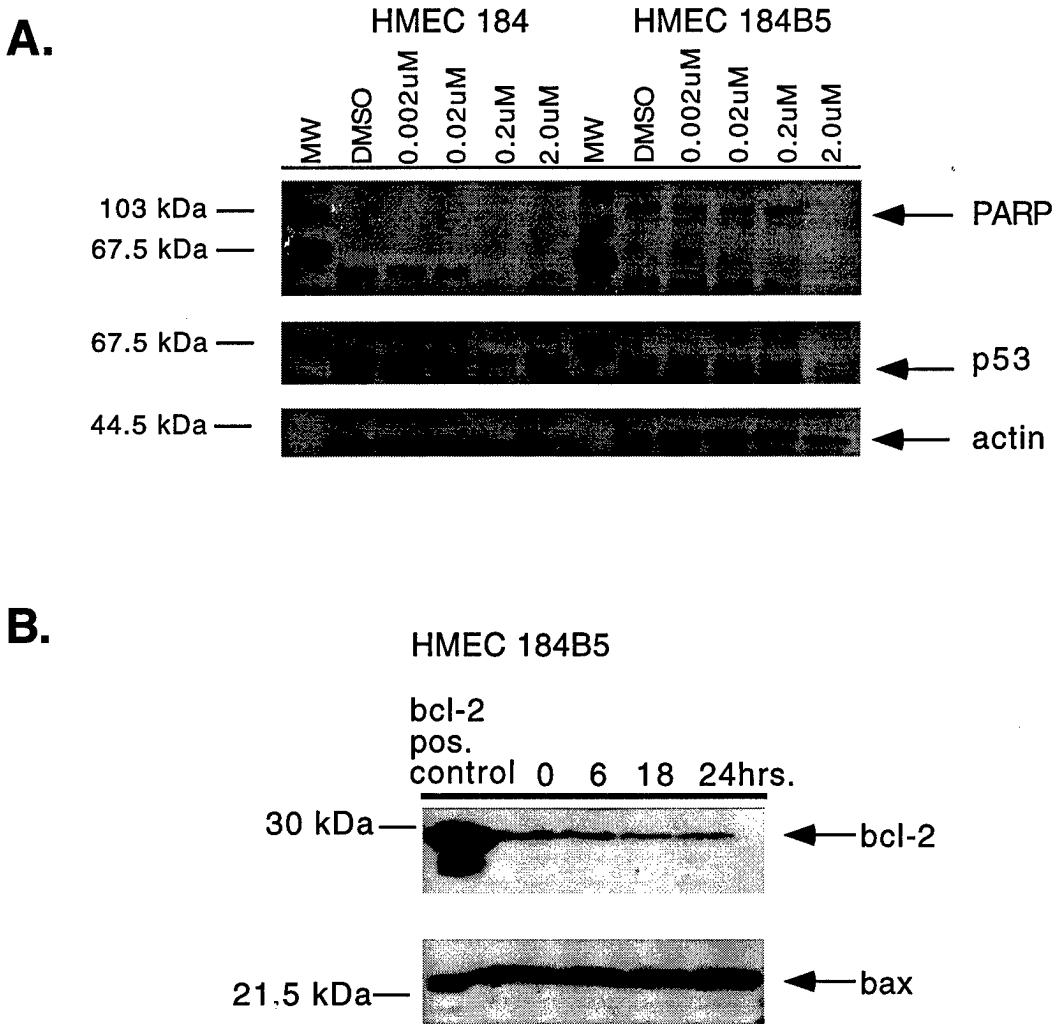


Figure 3. Western Blot analysis of apoptotic regulators in HMEC. **A**, HMEC 184 or HMEC 184B5 cells were exposed to various doses of staurosporine (SSP) for 24 hrs., then collected and 10ug of protein was separated on a SDS-PAGE gel, transferred to a PVDF membrane, then probed with antibodies against PARP, p53. β-actin is shown as a loading control. **B**, HMEC 184B5 cells were exposed to 2.0 uM staurosporine (SSP) for various times, then collected and 10ug of protein was separated on a SDS-PAGE gel, transferred to a PVDF membrane, then probed with antibodies against bcl-2 or bax.

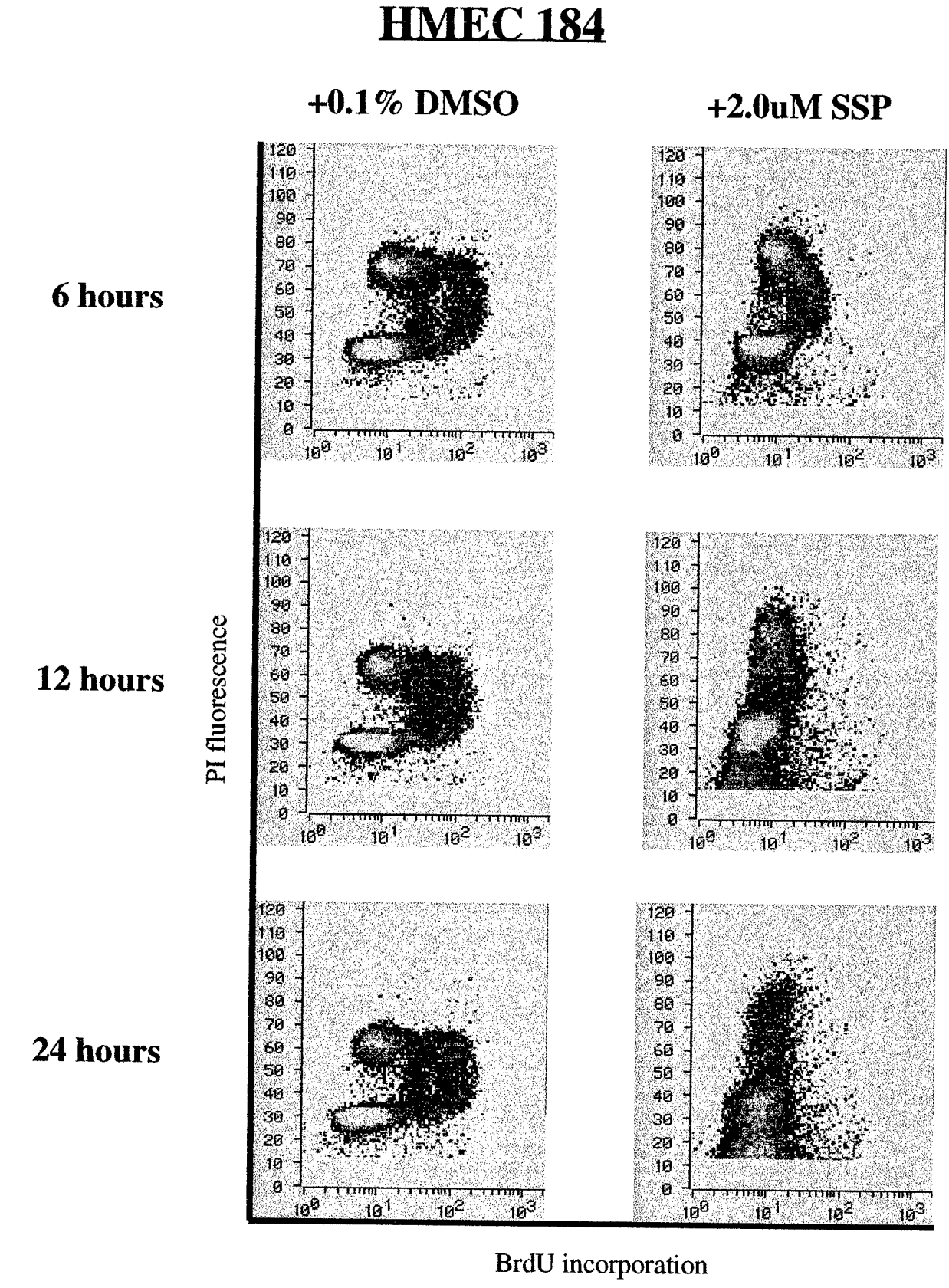


Figure 4. Dual-parameter FACS analysis of staurosporine treated HMEC 184 cells. HMEC 184 cells were exposed to vehicle (0.1% DMSO) or 2μM staurosporine (SSP) for various times, then collected and analyzed by dual-parameter FACS. The cells were pulsed with BrdU 15 min. prior to collection.

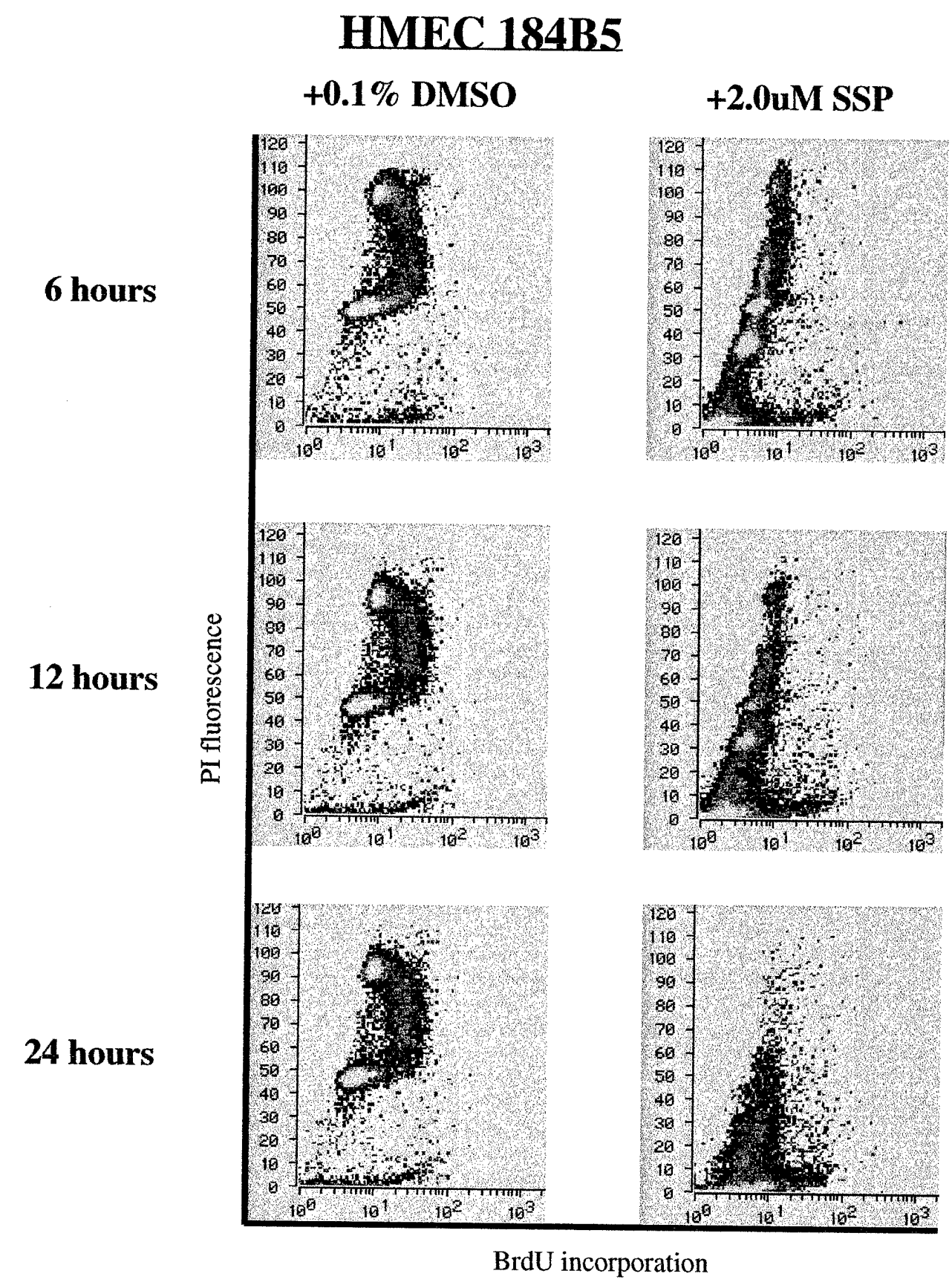


Figure 5. Dual-parameter FACS analysis of staurosporine treated HMEC 184B5 cells. HMEC 184B5 cells were exposed to vehicle (0.1% DMSO) or 2μM staurosporine (SSP) for various times, then collected and analyzed by dual-parameter FACS. The cells were pulsed with BrdU 15 min. prior to collection.

HMEC 184

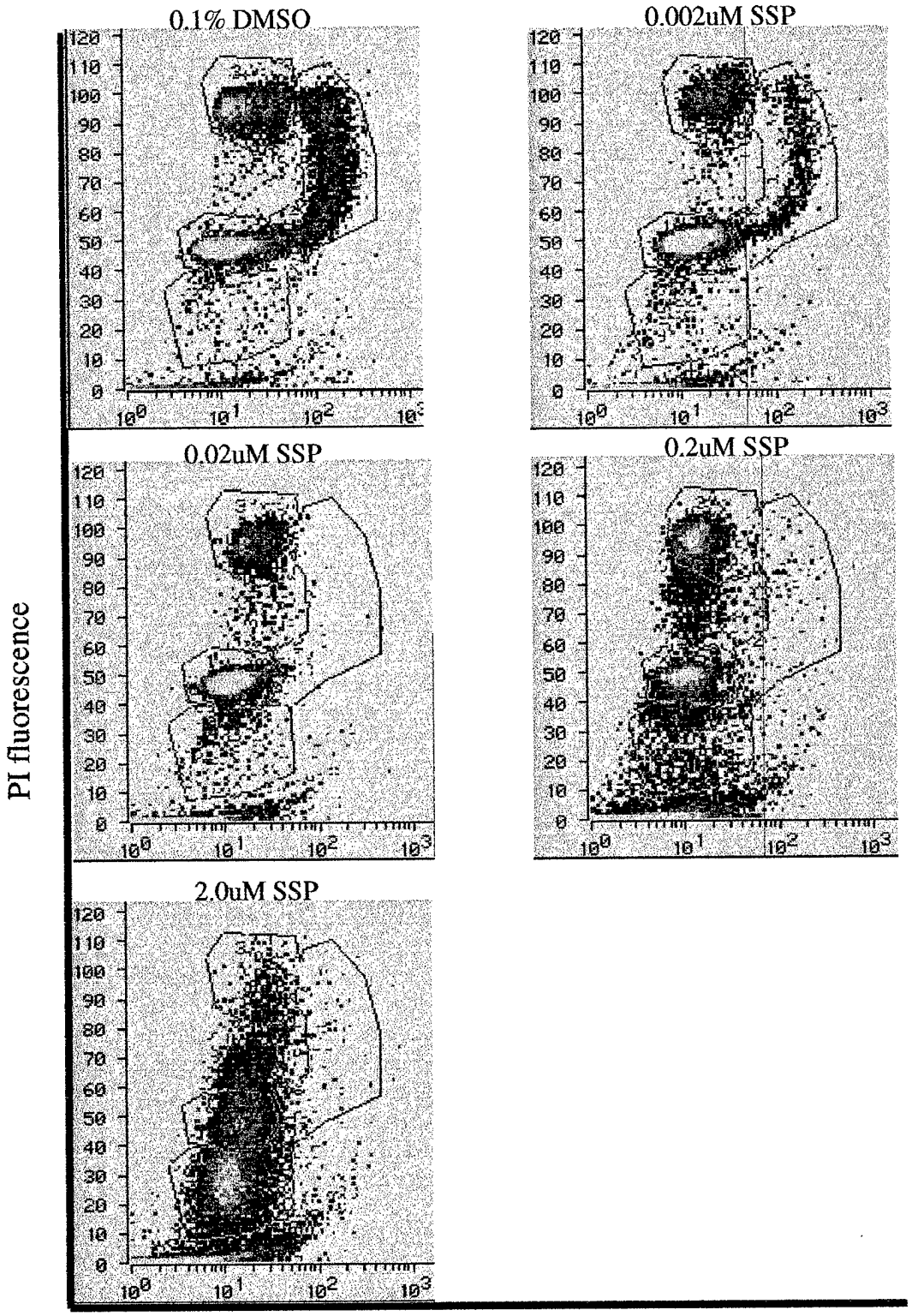


Figure 6. Dual-parameter FACS analysis of staurosporine treated HMEC 184 cells. HMEC 184 cells were exposed to vehicle (0.1% DMSO) or increasing doses of staurosporine (SSP) for 24 hours, then collected and analyzed by dual-parameter FACS. The cells were pulsed with BrdU 15 min. prior to collection.

HMEC 184B5

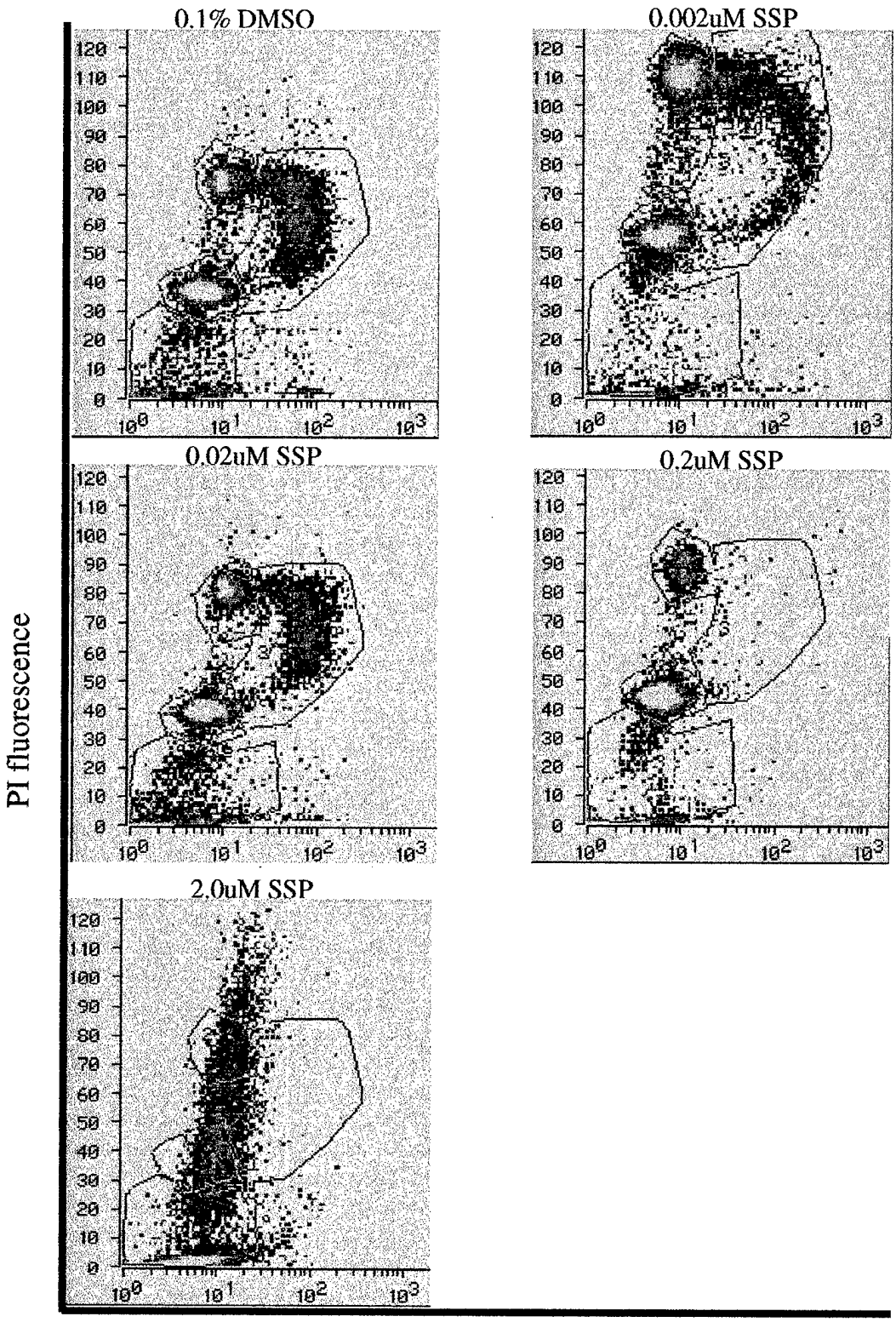


Figure 7. Dual-parameter FACS analysis of staurosporine treated HMEC 184B5 cells. HMEC 184B5 cells were exposed to vehicle (0.1% DMSO) or increasing doses of staurosporine (SSP) for 24 hours, then collected and analyzed by dual-parameter FACS. The cells were pulsed with BrdU 15 min. prior to collection.